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Note

Electrochemical detection for plasma catecholamines: apparent loss of dihydroxybenzylamine in dog plasma

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Electrochemical detection (ED) in conjunction with high-performance liquid chromatography (HPLC) has become a popular method for determination of plasma catecholamine concentrations. One commonly used internal standard for the procedure is dihydroxybenzylamine (DHBA). It has been suggested that the internal standard be added at the time of sample collection to account for any loss which occur during storage[1]. Recently, this laboratory has begun using HPLC–ED for measurement of catecholamines in dog plasma. During the course of these experiments, it was noted that DHBA seemed to "disappear" from plasma stored at −30°C for up to 2 weeks and also from samples stored at −80°C. Studies were thus performed to determine whether the apparent concentration of DHBA was indeed affected by storage in dog plasma and if so to what extent. Similar studies were also performed using human plasma.

EXPERIMENTAL

General

The chromatographic apparatus consisted of a Tracor 950 dual-piston pump with internal damper, Rheodyne injection port with a 100-μl loop, a 25-cm ODS 10 pre-column, a 25-cm ODS 5 column, a glassy carbon working electrode (Bioanalytical Systems), a Ag/AgCl reference electrode, an LC-4B amperometric detector (Bioanalytical Systems), and Tracor TS-10 recorder.

The mobile phase used consisted of 0.15 *M* monochloroacetate buffer, pH 3.0, containing 2 m*M* disodium EDTA and 75 mg/l sodium octyl sulfate. The flow-rate was 2.2 ml/min and the working electrode was maintained at a potential of 0.65 V.

Sample analysis

Sample analysis was as described by Bioanalytical Systems[2]. Briefly, to each plasma sample was added 50 mg activated aluminum oxide (AAO) and 1.0 ml Tris buffer. The sample was then vigorously shaken for 5 min, the supernatent removed and the AAO was rinsed 2× with distilled, demineralized water, and the AAO spun dry. Next, 200 μl of 0.1 *M* $HClO_4$ was added to the AAO, allowed to stand for 5 min, and then centrifuged 1 min to collect the $HClO_4$ which contained the catecholamines. A 50-μl volume of the acid was then injected into the HPLC–ED system.

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Experimental protocol

Dog plasma. The first experiment performed was to determine whether DHBA was absorbed onto the polypropylene tubes used for sample storage. The DHBA was solubilized in 0.1 *M* $HClO_4$ at a concentration of 100 ng/ml, and 100 μl were added to two polypropylene tubes. One sample was analyzed immediately and the second was allowed to stand at room temperature for 2.5 h before analysis. No other materials (*e.g.*, AAO, buffer, etc.) were added to the second tube until time of analysis.

The second series of experiments examined whether DHBA was lost during storage at either −30°C or −80°C for up to 12 days. Six blood samples (6 ml each) were drawn into clean syringes and immediately transferred into iced centrifuge tubes containing 100 μl of stabilizing solution. The stabilizing solution contained 90 mg/ml ethyleneglycol-bis(β-aminoethyl ester)-N,N′-tetraacetic acid (EGTA) and 60 mg/ml glutathione. The blood was then centrifuged at 0°C for 10 min. Exactly 2.0 ml of plasma were removed and placed in a polypropylene tube. To each of these samples were added 100 μl of DHBA and 50 μl of norepinephrine (NE) (75 ng/ml in 0.1 *M* $HClO_4$). Sample 1 was analyzed immediately, samples 2–5 were stored at −30°C, and sample 6 was stored at −80°C. Samples 2–5 were analyzed on days 2, 6, 9 and 12, respectively. Sample 6 was analyzed on day 12.

The third study examined the practicality of adding DHBA to all samples at one time on the day of analysis. Approximately 50 ml of plasma was collected from one dog, and 84 ng of NE was added to the plasma. From this pooled plasma, nineteen 2.0 ml samples were used. DHBA was added to 10 samples (numbered 1 through 10) at zero time and analyzed at 4–6-min intervals. The remaining 9 samples (numbered 2A through 10A) were run concurrently with samples 2–10, but the DHBA was added immediately before analysis. The total time interval was determined from the addition of DHBA to the time the Tris buffer was added to the sample.

Human plasma. To determine whether DHBA may react differently in human plasma than dog plasma, 30 ml of plasma were collected from a 34-year-old white female. To this pooled plasma were added 5 ng of NE (1.83 ng/ml plasma, final concentration). Samples 1–7 had DHBA added at time zero, and samples 2A–7A had DHBA added immediately before analysis. Each pair of samples was analyzed at 10-min intervals. Sample 8 also had DHBA added at time zero, and numbers 8 and 8A were stored at −30°C and analyzed 3 days later.

RESULTS AND DISCUSSION

The first study was to determine whether DHBA may adhere to, or be absorbed by, the polypropylene tubes used for plasma storage. When the two samples were analyzed 2.5 h apart, there was no difference in the peak heights obtained. Thus, these storage tubes do not affect the concentration of DHBA in the samples.

The second study examined the stability of DHBA at −30°C and −80°C for up to 12 days. Fig. 1 illustrates the effect of time on the peak heights obtained for NE and DHBA when the plasma samples were assayed on day 2, 6, 9 and 12 at either temperature. It is clear that the apparent concentration of DHBA was erratic and unpredictable. Further, during storage at −80°C the peak height for DHBA decreased from 70 mm to 35 mm. The peak heights for NE were essentially un-

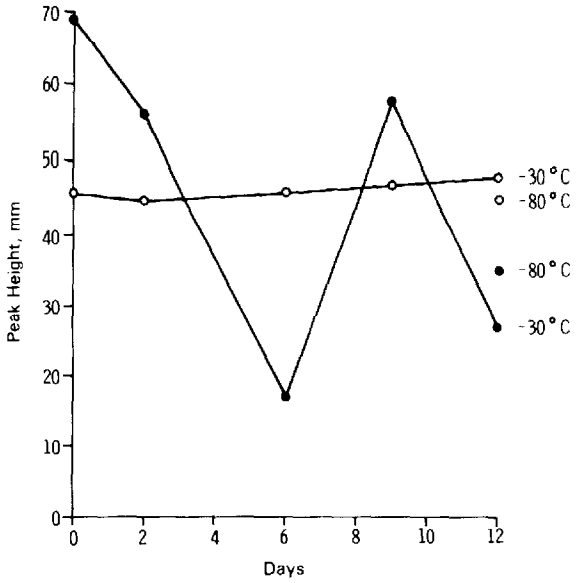

Fig. 1. Peak heights for NE (○) and DHBA (●) when dog plasma samples were stored at −30°C or −80°C.

changed, beginning at 46 mm and ending at 48 mm for −30°C and 45 mm for −°C. Table I lists the calculated values for NE concentrations using these peak heights. As can be seen, there is a 4-fold variation in these calculated values due to the erratic values obtained for DHBA.

The third study examined whether the apparent DHBA concentration was affected within the time needed to assay 10 plasma samples. Fig. 2 illustrates the calculated NE values for two series of samples. When DHBA was added to all 10 samples at one time, there was a progressive increase in the calculated NE value from 1.55 ng/ml up to 3.87 ng/ml at 55 min. Even under the hurried condition of assaying 19 samples within 1 h, the samples which had DHBA added immediately before addition of Tris buffer were remarkably consistent with a mean value of 1.59 ng/ml and a coefficient of variation of 4.7%. Thus, the addition of DHBA to all samples at once can lead to erroneous calculations.

When a similar series of experiments were performed using human plasma, there was no apparent loss of DHBA noted. When DHBA was added to plasma at time zero and each sample analyzed at 10-min intervals, the mean value for all 7 samples was 2.24 ng/ml (S.D. = 0.17 and coefficient of variation, C.V. = 7.6%). In the samples which had DHBA added immediately before analysis, the mean was 2.14 ng/ml (S.D. = 0.10 and C.V. = 4.9%). Thus, there was no difference in the values obtained using either of the two methods for adding DHBA to human plasma samples. When two samples were stored at −30°C for 3 days, the sample which contained DHBA had a calculated NE concentration of 2.42 ng/ml and the one which had DHBA added immediately before analysis had a NE value of 2.38 ng/ml. Again, there was no difference between these values, nor were they different from the values obtained 3 days earlier.

TABLE I

CALCULATED VALUES FOR PLASMA NOREPINEPHRINE WHEN DHBA ADDED ON DAY 0 AND SAMPLES STORED AT −30°C OR −80°C

Day	*Storage temperature (°C)*	*NE (ng/ml)*
0	–	2.32
2	−30	2.34
6	−30	8.99
9	−30	2.58
12	−30	6.33
12	−80	4.58

The differences noted between the calculated values for NE and the 1.83 ng/ml NE added reflect an endogenous value of approximately 0.4 ng/ml NE.

The reason for the apparent loss of measurable DHBA in dog plasma is not clear but most likely is the result of irreversible protein binding which does not happen with NE, nor does it occur with human plasma. One bit of evidence which would support the theory of protein binding is that the standard solutions for extraction which are made daily with phosphate buffer (instead of plasma) did not produce changes in DHBA. The peak height ratio of NE mm/DHBA mm for the standard which contained DHBA for 60 min before the addition of Tris buffer was 0.522 compared to 0.525 for the standard which was assayed at time zero.

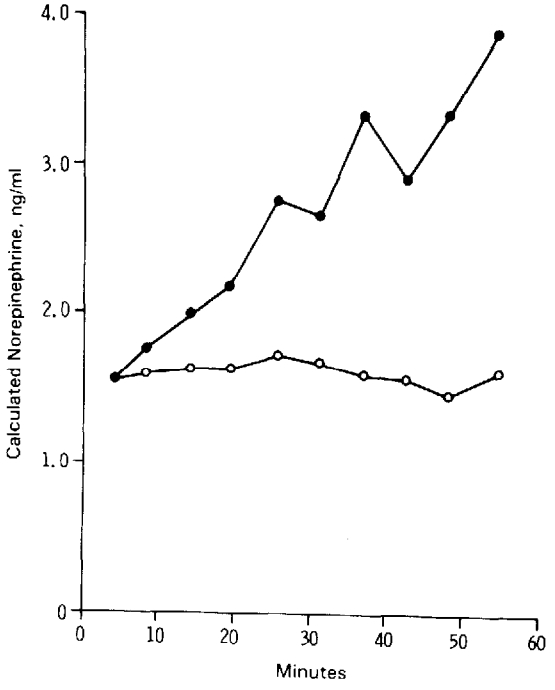

Fig. 2. Calculated values for NE when DHBA added either at time of assays (○) or at 0 min (●).

In conclusion, when catecholamines are measured in dog plasma, the internal standard DHBA should be added immediately before the addition of Tris buffer in the assay procedure, because DHBA apparently binds to some factor in dog plasma thereby giving erroneously high values for NE concentration. Samples of dog plasma should not have DHBA added before storage even at −80°C for the same reasons. This "loss" of DHBA does not occur in human plasma stored at −30°C for 3 days.

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